5732–5742 *Nucleic Acids Research, 2016, Vol. 44, No. 12* *Published online 25 April 2016*
doi: 10.1093/nar/gkw302

Mitochondrial transcription termination factor 1 directs polar replication fork pausing

Yonghong Shi[1,2,†], Viktor Posse[1,†], Xuefeng Zhu[1,2], Anne K. Hyvärinen[3], Howard T. Jacobs[3,4], Maria Falkenberg[1,*] and Claes M. Gustafsson[1,*]

[1]Institute of Biomedicine, University of Gothenburg, P.O. Box 440, SE-405 30 Gothenburg, Sweden, [2]Center for Molecular Medicine, National Heart Lung and Blood Institute, NIH, Bethesda, MD 20892, USA, [3]BioMediTech and Tampere University Hospital, FI-33014, University of Tampere, Finland and [4]Institute of Biotechnology, FI-00014, University of Helsinki, Finland

Received February 17, 2016; Revised April 11, 2016; Accepted April 12, 2016

ABSTRACT

During replication of nuclear ribosomal DNA (rDNA), clashes with the transcription apparatus can cause replication fork collapse and genomic instability. To avoid this problem, a replication fork barrier protein is situated downstream of rDNA, there preventing replication in the direction opposite rDNA transcription. A potential candidate for a similar function in mitochondria is the mitochondrial transcription termination factor 1 (MTERF1, also denoted mTERF), which binds to a sequence just downstream of the ribosomal transcription unit. Previous studies have shown that MTERF1 prevents antisense transcription over the ribosomal RNA genes, a process which we here show to be independent of the transcription elongation factor TEFM. Importantly, we now demonstrate that MTERF1 arrests mitochondrial DNA (mtDNA) replication with distinct polarity. The effect is explained by the ability of MTERF1 to act as a directional contrahelicase, blocking mtDNA unwinding by the mitochondrial helicase TWINKLE. This conclusion is also supported by *in vivo* evidence that MTERF1 stimulates TWINKLE pausing. We conclude that MTERF1 can direct polar replication fork arrest in mammalian mitochondria.

INTRODUCTION

Human mitochondrial DNA (mtDNA) is a circular, double-stranded molecule. The two strands differ in their buoyant density in alkaline cesium chloride gradients and they are therefore called the light strand (L-strand) and the heavy strand (H-strand), respectively (1). Human mtDNA encodes 13 components of the respiratory chain. The double-stranded genome also produces 22 tRNAs and 2 rRNAs needed for mitochondrial translation. Transcription is initiated from the heavy- and light-strand promoters (HSP and LSP), which generate polycistronic, near full genome-sized transcripts covering the two strands. The primary transcripts are then processed to produce individual RNA molecules (2).

The molecular machineries responsible for transcription and replication of mammalian mtDNA are distinct from those found in the nucleus. For instance, DNA synthesis is performed by the heterotrimeric DNA polymerase γ (POLγ), which consists of a catalytic subunit (POLγA) and two identical accessory subunits (POLγB) (3,4). The replicative mtDNA helicase TWINKLE is a homohexameric protein complex with similarity to the bacteriophage T7 primase/helicase gene 4 protein (T7 gp4), but without the primase activity present in the phage protein (5–8). A mitochondrial single-stranded DNA binding protein (mtSSB) protects the single-stranded parental strand during mtDNA replication and stimulates both POLγ and TWINKLE activities (6,9–12). Mitochondria also contain a specialized transcription machinery, which include a monomeric RNA polymerase (POLRMT) and two accessory factors, mitochondrial transcription factors A and B2 (13–16). There is also a mitochondrial transcription elongation factor (TEFM), which helps POLRMT to transcribe longer stretches of RNA, and to bypass regions generating highly structured RNA (17–19). In addition to producing mRNA, tRNA and rRNA, the mitochondrial transcription machinery also generates primers for initiation of mtDNA replication at the major origins of the two strands (20).

For a long time, it was believed that mitochondrial transcription is regulated at the level of termination. This idea was based on the identification of the MTERF1 (formerly denoted as mTERF), a protein that binds sequence-specifically to a 28-bp region immediately downstream of

*To whom correspondence should be addressed. Tel: +46 31 7863826; Fax: +46 31 416108; Email: claes.gustafsson@medkem.gu.se
Correspondence may also be addressed to Maria Falkenberg. Tel: +46 31 7863444; Fax: +46 31 416108; Email: maria.falkenberg@medkem.gu.se
†These authors contributed equally to the work as first authors.

the 16S rRNA gene (21,22). According to the belief at the time, MTERF1 regulated the levels of heavy-strand transcription that proceeded beyond its binding site, a model that was put forward to explain why rRNA transcripts were almost 50 times more abundant than the mRNA transcripts produced downstream of the MTERF1 binding site (23). More recently, *in vivo* analysis of cultured human cells (24) and the *Mterf1*$^{-/-}$ knockout mouse refuted this idea (25). Loss of MTERF1 does not affect the relative steady-state levels of rRNAs and mRNA encoded on the H-strand. Instead, these relative RNA levels are most likely explained by differences in stability, with rRNA having a much longer half-life. Studies of the *Mterf1*$^{-/-}$ knockout mouse model instead revealed that Mterf1 prevents antisense transcription from entering the rRNA gene region. In the absence of Mterf1 antisense transcription progresses beyond the rRNA genes and may also interfere with light-strand promoter (LSP) function. These *in vivo* findings agree nicely with the effects of MTERF1 on transcription termination *in vitro*, which found that the protein completely blocks L-strand transcription, but only has a minor effect on H-strand transcription (26,27).

According to the strand-asymmetric model of mtDNA replication, DNA synthesis is continuous on both strands and each strand contains a distinct, major origin of replication, OriH and OriL (1,28). Replication is strand-asynchronous, i.e. synthesis of the H-strand is first initiated at OriH and the reaction proceeds approximately two thirds of the way around the circular genome, displacing the non-template H-strand, which becomes transiently complexed with mtSSB (29). Two-dimensional agarose gel-electrophoresis has also identified the presence of RNA annealed to the displaced, non-template H-strand DNA (30,31). It has been proposed that these RNA species can replace mtSSB and stabilize ssDNA during replication, but this remains a debated issue (29,32). L-strand DNA synthesis is initiated at OriL by a process that is believed to involve activation of the origin when it becomes single-stranded. The main features of this strand-asymmetric replication mode are supported by a number of different observations, including electron microscopy analysis (28), biochemical analysis of replication intermediates in both wild-type cells and tissues, and where expression of key replication factors has been manipulated (33–35), *in vivo* saturation mutagenesis (36), the *in vivo* occupancy pattern of mtSSB (29), and the possibility to reconstitute specific steps of this process *in vitro* (12,20,37).

How DNA replication and transcription are coordinated in human mitochondria is not known. Unregulated clashes between replication and transcription may affect gene transcription and/or impair DNA replication (38). To avoid such problems in nuclear DNA replication, the moving replication fork can be arrested close to the 3′-end of highly transcribed ribosomal DNA (rDNA). A replication fork barrier prevents DNA replication from progressing in a direction opposite that of rDNA transcription, which supposedly prevents head-on collisions between replication and transcription machineries (38). In mouse, the mTTF-1 protein binds to Sal boxes situated just downstream of the ribosomal transcription units and block replication with opposite polarity to that of ribosomal transcription (39). In budding yeast, the Fob1 protein ensures that rDNA replication only occurs in the direction of transcription and that the replication machinery does not disturb transcription of rDNA (40). If a similar replication fork barrier protein also exists in mitochondria is not known, but the binding of MTERF1 just downstream of the 3′-end of rDNA makes it an interesting candidate for such a function. In support of this idea, a previous report demonstrated that overexpression of MTERF1 stimulates replication pausing at MTERF1 binding sites *in vivo*. The expression levels of MTERF1 correlated with the strength of the pausing and it was hypothesized that MTERF1 acts to coordinate the passage of replication and transcription complexes (41). In this report, we use *in vitro* biochemistry to demonstrate that MTERF1 acts as a replication fork barrier and delays replication fork progression. The effect is explained by MTERF1's ability to work as a contrahelicase with distinct polarity.

MATERIALS AND METHODS

Recombinant proteins

TWINKLE, mtSSB, POLγA, POLγB, and MTERF1 were expressed and purified as described previously (6,12,27,42). POLRMT, TFAM, TFB2M and TEFM were expressed and purified as described in (19).

In vitro transcription experiments

The HSP and LSP transcription templates were cloned as described in (13) and linearized with PstI and EcoRI respectively. The transcription reaction volumes were 25 μl and contained 25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 40 mM NaCl, 100 μg/mL BSA, 10 mM DTT, 400 μM ATP, 150 μM GTP, 150 μM CTP 10 μM UTP, 0,02 μM α-^{32}P UTP (3000 Ci/mmol), 4 U RNase inhibitor Murine (New England Biolabs), 4 nM of indicated plasmid template. The reactions contained POLRMT (20 nM), TFAM (200 nM), and TFB2M (60 nM). TEFM (40 nM) and MTERF1 (2, 8 and 32 nM) were added as indicated. The reactions were stopped and analyzed on 4% denaturating polyacrylamide gels as described previously (13).

Templates preparation

We cloned a DNA fragment corresponding to nt 3056–3559 of the mitochondrial human genome containing the MTERF1 binding site (nt 3231–3253) between the HindIII and EcoRI sites in the pBluescript SK(+) and pBluescript SK(−) vectors (Agilent Technologies; La Jolla, CA, USA). The pBluescript SK(−) MTERF1 construct was used as a template for site-directed PCR mutagenesis reactions to generate a mutant variant of the MTERF1 binding site containing a 4-bp deletion (nt 3241–3244). All constructs were confirmed by sequencing and used to isolate ssDNA following the manufacturer's protocol (Stratagene). To produce the rolling-circle DNA replication templates, we annealed a 70-mer oligonucleotide (5′-40[T]- AGT TAC CAA TGC TTA ATC AGT GAG GCA CCT-3′) to the pBluescript SK(+) and (5′-40[T]-TCG CTG AGA TAG GTG CCT CAC TGA TTA AGC-3') to the pBluescript SK(−)

ssDNA (7.5 pmol) and synthesized the second strand as described previously (20).

To prepare the helicase substrates indicated in the figure legends, three different oligonucleotides; Helicase (WT) forward; TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT AAG ATG GCA GAG CCC GGT AAT, Helicase (WT) reverse; TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT ATT ACC GGG CTC TGC CAT CTT A, or Helicase (del) reverse; TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TCG ATT ACC GGG GCC ATC TTA AC were labeled with ^{32}P at the 5′-termini using T4 polynucleotide kinase (Stratagene) and annealed to ssDNA (described above) containing the corresponding DNA sequence creating the helicase substrates indicated in the figure legends. After annealing, the samples named SK+Terf1, SK-Terf1, and SK- TERF1 del were run through Centricon 100 columns (Millipore Company) to remove unannealed oligonucleotide as previously described (6).

Rolling-circle mtDNA replication

The reaction mixtures (20 μl) contained 10 fmol of the indicated dsDNA template and were performed as described previously but in the presence of MTERF1 when indicated in the figure legends. The reactions were incubated at 37°C and stopped after 40 min (or at the times indicated) by adding 200 μl of transcription stop buffer, 0.5% SDS and 2 μl of proteinase K. After another 45 min incubation at 42°C, the samples were precipitated and analyzed as described before (12).

Helicase assays

DNA templates for TWINKLE helicase assays were prepared as described above. The reaction mixture (25 μl) contained 20 fmol of the indicated DNA substrate, 20 mM Tris–Cl (pH 7.6), 4.5 mM $MgCl_2$, 10 mM DTT, 3 mM UTP, 0.1 mg/ml BSA, 40 mM NaCl, 600 fmol TWINKLE or 100 fmol T7 gp4 protein and 500 fmol of MTERF1 if nothing else is indicated in the figure legends. The reactions were incubated at 32°C for 20 min (or as indicated) and stopped by the addition of 2 μl stop buffer (90 mM EDTA, 6% SDS, 30% glycerol, and 0.25% bromphenol blue), and analyzed on a 6% non-denaturing polyacrylamide gel.

Chromatin immunoprecipitation analysis

MTERF1-MycHis expressing Flp-In™ T-RexTM-293 cells (41) were cultured in DMEM medium with 5% fetal bovine serum, *hygromycin* B (50 μg/ml) and blasticidin-HCl (15 μg/ml) in 10 × 75 cm^2 tissue culture flasks to 80% confluence. Of these, 5 × 75 cm^2 flasks cells were induced to express the Myc-tagged mTERF transgene by addition of 100 ng/ml doxycycline, whereas the other 5 × 75 cm^2 flasks were kept as a non-induced control. After 24 h induction, the cells were harvested and washed once with ice-cold phosphate buffered saline (PBS). Isolation of mitochondria was carried out following a protocol described in (43). The mitochondria were washed once with ice-cold PBS, and next incubated with 1% formaldehyde in PBS for 10 min at rt. The crosslinking reaction was quenched by addition of glycine (final conc. 125 mM) and then incubated for five additional minutes. After washing twice in ice-cold PBS, mitochondria were lysed in 25 mM HEPES-KOH (pH 7.6), 10% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5% tween-20, 0.15 M KCl, 1 mM phenylmethlsulfonylfluoride, 2 mM pepstatin A, 0.6 mM leupeptin, and 2 mM benzamidine. The mitochondrial lysates were sonicated in a Bioruptor UCD 200TM (Diagenode) for 10 min at high output, with intervals of 30 s on and 30 s off, and then centrifuged for 5 min at 14 000 × g. Aliquots of 100 μl wt and induced supernatant were incubated with 1.5 μl of human TWINKLE polyclonal antibody (Agrisera, Sweden) or 1.5 μl rabbit IgG (ab37415, Abcam) overnight in a rotator at 4°C. 50 μl of protein A beads (GE healthcare) were added to supernatants and the indicated antibody for 1 h at 4°C. After wash and elution, samples were incubated overnight at 65°C to reverse crosslinking. RNA contaminations were removed by incubation with 100 ng/ml RNaseA for 15 min at 37°C, and proteins were removed by addition of 20 μg proteinase K and incubation for 2 h at 56°C. DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation. The purified DNA was used for real-time PCR analysis (Bio-Rad) with the primer pairs listed in Supplementary Table S1. Quantifications were performed using real time PCR Software (Bio-Rad) and Excel (Microsoft); ratios of IP/input are depicted in the figures after subtracting ratios obtained from the rabbit IgG control. Three independent biological replicates were carried out for ChiP analysis. Averages and standard deviations were calculated and plotted in Microsoft Excel.

RESULTS

MTERF1 effectively terminates transcription in the presence of TEFM

The transcription elongation factor TEFM was just recently identified (17–19). Before proceeding to studies of MTERF1 and DNA replication, we decided to investigate if the presence of TEFM affected MTERF1 activities *in vitro* and conclusions drawn from these previous studies (27). In mitochondria, transcription can approach the MTERF1 binding site from two different directions. To mimic each of these situations, we used linearized DNA templates with either the HSP or LSP promoter, followed by the MTERF1 binding site oriented in the same direction relative the promoter as that observed *in vivo* (Figure 1A). In our analysis, we added increasing concentrations of MTERF1 to transcription reactions containing POLRMT, TFAM, TFB2M, and the DNA template. Using the LSP template in the absence of MTERF1 we observed synthesis of the expected full-length run-off transcripts (Figure 1B, lane 1, LSP RO) and also a previously described pre-terminated transcript at CSBII (CSBII PT). When we added increasing amounts of MTERF1, we observed the appearance of an additional transcript with the expected size for an RNA transcript prematurely terminated at the MTERF1-binding site (Figure 1B, lanes 2–4, MTERF1 PT). In agreement with previous reports, addition of TEFM stimulated transcription and abolished transcription termination at CSBII. However, it

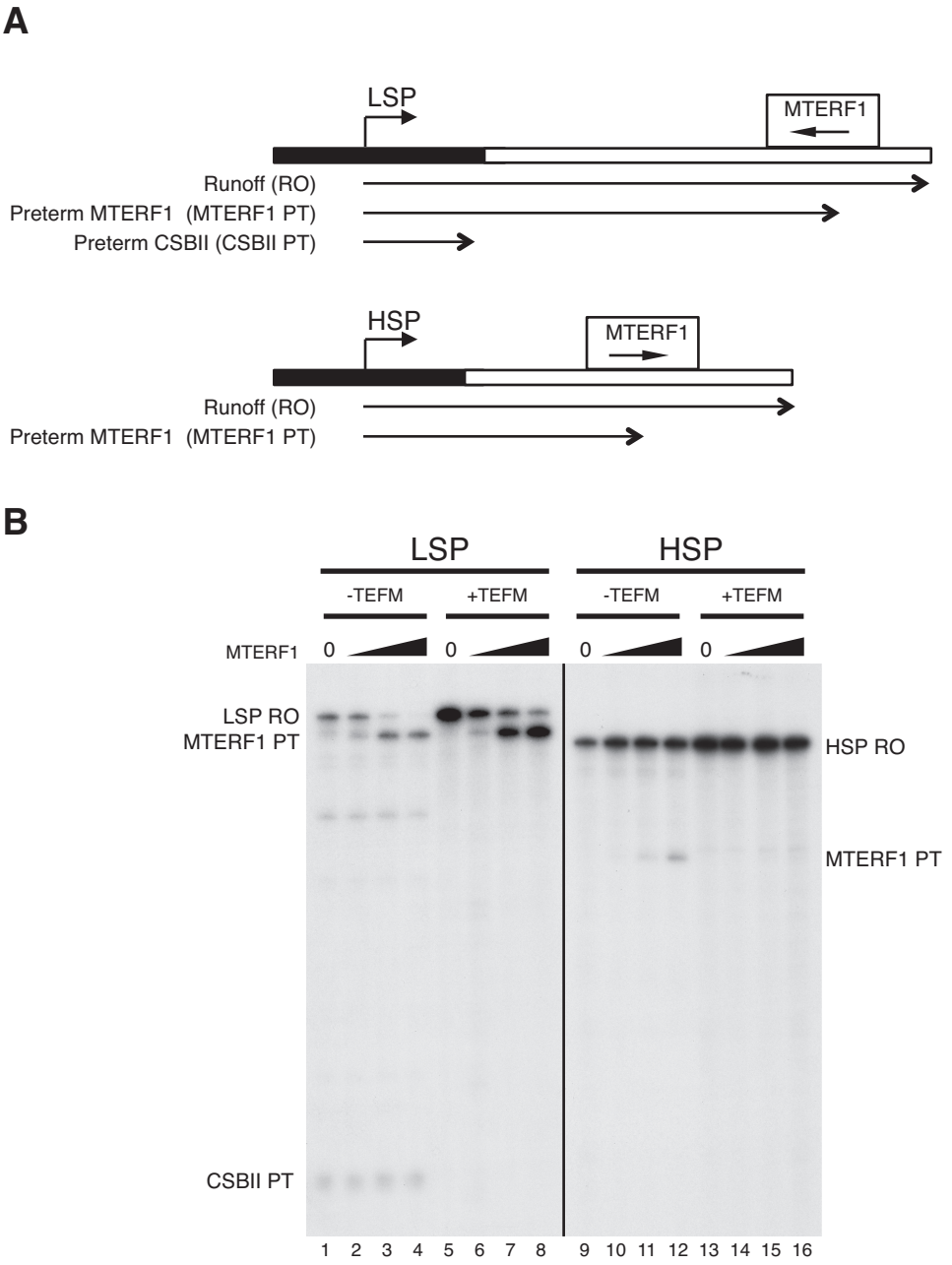

Figure 1. MTERF1 effects on transcription. (**A**) A schematic description of the templates used. The arrows indicates the direction of the MTERF1 binding site relative the transcription machinery. (**B**) *In vitro* transcription with the recombinant transcription machinery (POLRMT, TFAM and TFB2M) on MTERF1 binding site-containing templates, with transcription approaching MTERF1 either in the LSP direction (lanes 1–8) or the HSP direction (lanes 9–16). The respective promoter has been used in these experiments (LSP for lanes 1–8 and HSP for lanes 9–16). The bands corresponding the runoff products (LSP RO and HSP RO), as well as the premature transcription terminations at CSBII (CSBII PT) and the MTERF1 binding site (MTERF1 PT) are indicated. TFEM was added to reactions in lanes 5–8 for LSP and lanes 13–16 for HSP.

did not affect MTERF1-dependent transcription termination (Figure 1B, lanes 5–8).

We next analyzed effects on the HSP template. In the absence of MTERF1 we observed synthesis of the expected full-length run-off transcripts (Figure 1B, lane 9, HSP RO). Addition of increasing concentrations of MTERF1 was much less effective in terminating transcription in this orientation and we only observed the appearance of a very weak band at the expected size for an RNA transcript terminated at the MTERF1-binding site (Figure 1B, lanes 10–12, MTERF1 PT). Addition of TEFM abolished this weak band (Figure 1B, lanes 13–16). From our observations, we conclude that MTERF1 terminates transcription initiated from LSP also in the presence of TEFM. In contrast, TEFM does decrease the already very weak MTERF1 termination activity on transcription initiated from HSP.

MTERF1 pauses mtDNA replication in an orientation-dependent manner *in vitro*

We next addressed the effects of MTERF1 on mtDNA replication. The purified POLγ, mtSSB, and TWINKLE proteins together form a minimal mitochondrial replisome, which in rolling circle replication can support synthesis of single-stranded DNA stretches of more than 20 000 nt (12). We constructed a DNA template that contained a replication fork on which the replication machinery could be loaded. The template also contained an approximately 3.5 kb long dsDNA region and a free 3′-end that can prime leading-strand DNA synthesis. On this type of circular template, leading-strand DNA synthesis coupled to continuous unwinding of the double-stranded template can, in principle, progress indefinitely. To monitor the effects of MTERF1, we introduced a 38-bp sequence covering the MTERF1 region, in two different orientations (Figure 2A).

First, we incubated the circular templates with the components of the mtDNA replication machinery and monitored DNA synthesis using radiolabeled dNTPs in a rolling-circle experiment (Figure 2B). To test if MTERF1 has a replication fork-barrier activity, we added increasing amounts of MTERF1 and monitored effects on DNA synthesis. When the MTERF1 site was positioned in the forward orientation (replication progressing in the same orientation as rRNA transcription initiated at HSP), we could not detect a significant effect on rolling-circle replication (Figure 2B, lanes 6–10). In contrast, when the MTERF1 site was in the reverse orientation (replication progressing in the opposite orientation to rRNA transcription, i.e. antisense transcription initiated at LSP), we observed a dramatic decrease in DNA synthesis (Figure 2B, lanes 1–5). These results indicated that MTERF1 blocks rolling-circle DNA replication in a direction-dependent manner.

Addition of MTERF1 not only led to inhibition of DNA replication, but also to the formation of replication products of defined lengths (e.g. Figure 2B, lane 5). To further elucidate this effect, we performed a time-course experiment. We used the rolling-circle template with the MTERF1 site oriented in the reverse direction. We incubated this template with our reconstituted *in vitro* DNA replication system in the presence of constant amounts of MTERF1 (Figure 2C). In the absence of MTERF1, the replication products formed a continuous smear (Figure 2C, lanes 1–4). In the presence of MTERF1 protein, we observed the formation of specific-sized replication products (Figure 2C, lanes 6–9) The size intervals between neighbouring replication products were constant, about 3.5 kb, which corresponded to the size of the DNA template molecule, suggesting that the replication fork arrested in the same region each time the circle was traversed. In addition, the first band was at about 5.5 kb, corresponding to the size expected for replication pausing at the MTERF1 binding site during the first such cycle. We next repeated the experiment with an identical template, but with the MTERF1 binding site oriented in the forward direction. With this template, we failed to observe any effects of the MTERF1 protein on DNA synthesis (Figure 2D). Our results support the conclusion that MTERF1 arrests the mtDNA replication machinery when approaching the 3′-end of the rRNA transcription unit, with the same directionality as transcription from LSP. The time-course experiment also demonstrated that mtDNA replication was not definitively terminated at the MTERF1 binding site, but that MTERF1 stimulated pausing at this site, since shorter replication products were chased into longer products over time (Figure 2C, compare 5.5 kb product in lanes 7–9).

To verify the relevance of MTERF1 binding for the observed effects on mtDNA replication, we prepared a rolling circle replication template with 4-bp deletion in the MTERF1 binding site (Figure 3 and Supplementary Figure S1). The effects of MTERF1 on mtDNA replication were monitored in a time-course experiment. The binding site mutation completely abolished the fork-pausing effect of MTERF1.

MTERF1 is a contrahelicase

POLγ is strictly dependent on TWINKLE for replication using a dsDNA template. We decided to monitor if MTERF1 could function by blocking TWINKLE-dependent DNA unwinding. To this end, we created DNA helicase substrates by annealing oligonucleotides ^{32}P-labeled in the 5′-end (for sequences, see Materials and Methods) to the complementary region of pBluescript II SK single-stranded DNA (SK+ or SK−) to form helicase substrates with a short double-stranded region and a 40-nt 5′-single-stranded tail. The double-stranded region contained the MTERF1-binding site in the forward or reverse orientation relative to the 40-nt 5′-single-stranded tail. In the absence of MTERF1, the TWINKLE helicase was able to unwind the DNA substrates. Addition of increasing amounts of MTERF1 had no effect on DNA unwinding of the template with the MTERF1 binding site in the 'forward' orientation (Figure 4A, lanes 1–6). In contrast, increasing amounts of MTERF1 dose-dependently blocked DNA unwinding of the template with the MTERF1 binding site in the reversed orientation (Figure 4A, lanes 7–12). We concluded that the ability of MTERF1 to block the TWINKLE DNA helicase is dependent of the relative orientation of the MTERF-binding site, since we observed a contrahelicase activity when unwinding progressed in an orientation opposite that of rRNA transcription, but when we reversed the orientation of the binding site, the contrahelicase activity was lost.

We next performed a time-course experiment with constant amounts of MTERF1. We used the fork-like helicase substrates with the double-stranded region containing a binding site for MTERF1 in the reverse direction (Figure 4B, lanes 1–10). We also included a substrate in which the MTERF1 binding site had been mutated by deleting 4 bp, AGAG, in the middle of the MTERF1 binding site (Figure 4B, lanes 11–20 and Supplementary Figure S1). As expected, MTERF1 was able to block TWINKLE DNA unwinding on the wild-type binding site template (Figure 4B, lanes 7–10). The effect was abolished when the MTERF1 binding site was mutated (Figure 4B, lanes 17–20). To facilitate further analysis and to investigate if the MTERF1 contrahelicase activity was specific to TWINKLE, we also investigated MTERF1 effects on a related DNA helicase, the bacteriophage T7 gene 4 protein (gp4) (Figure 4C). The MTERF1 and gp4 protein concentrations were kept

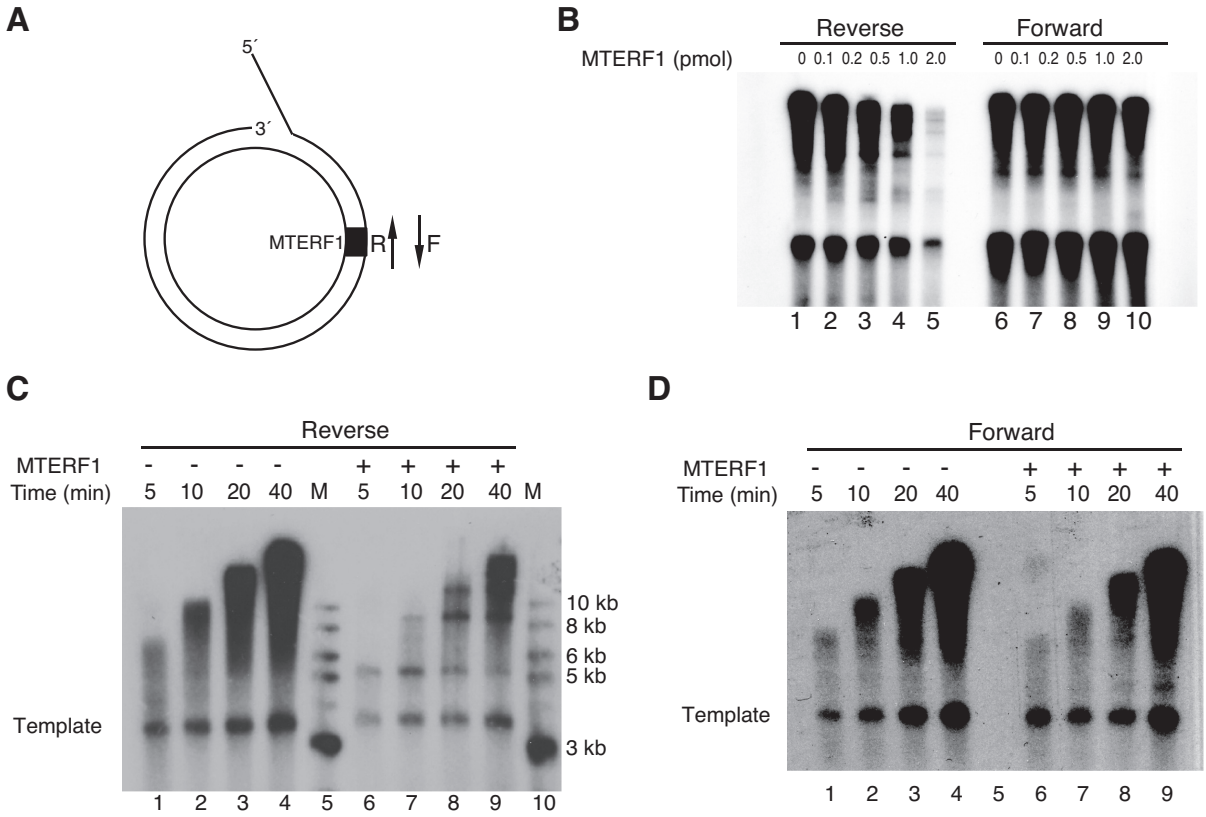

Figure 2. Effects of human recombinant MTERF1 on mtDNA replication *in vitro*. The preparation of the rolling circle templates and the replication reactions were performed as described in 'Materials and Methods'. (**A**) Schematic presentations of the DNA templates; the arrows show the direction of MTERF1 binding sites. (**B**) Polar pausing activity of recombinant human MTERF1 on mtDNA replication. Increasing amounts of MTERF1 were added as indicated and pausing was only observed when replication approached MTERF1 in the direction reverse to that of expected rDNA transcription. (**C**) Time curve of replication with MTERF1 binding site-containing template in reverse direction. Lanes 1–4 were reactions done in the absence of MTERF1 and lanes 6–9 in the presence of MTERF1 (750 fmol). Lanes 5 and 10 contains a labeled 1 kb DNA ladder with sizes indicated on the right. (**D**) Time curve of replication with MTERF1 binding site-containing template in forward direction. Lanes 1–4 are reactions in the absence of MTERF1 and lanes 6–9 in the presence of MTERF1 (750 fmol).

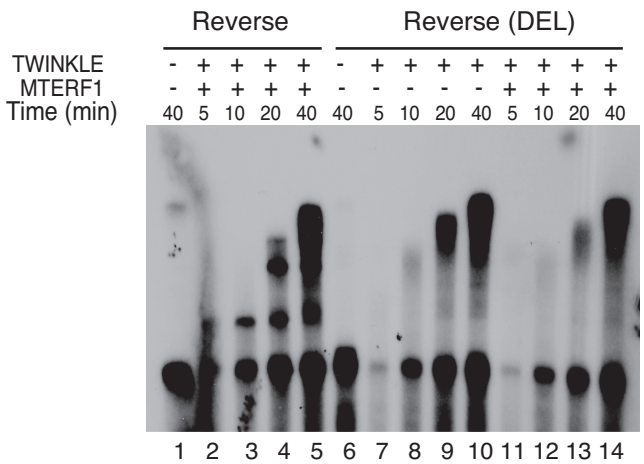

Figure 3. MTERF1 dependent pausing of mtDNA replication *in vitro* is dependent on the MTERF1 binding site. *In vitro* replication with rolling circle templates containing a wt MTERF1 binding site in the reverse direction (lanes 1–5) or a MTERF1 binding site with a 4-bp deletion in a reverse direction (lanes 6–14).

constant and we again monitored DNA unwinding of the template in a time course experiment. In the absence of MTERF1, the gp4 helicase could unwind both templates (Figure 4C, lanes 3–6 and 13–16). Addition of MTERF1 blocked gp4-dependent unwinding of the substrate containing the MTERF1 binding site in the reverse orientation (Figure 4C, lanes 7–10). MTERF1 did not affect the gp4-driven unwinding reaction, when the binding site was mutated (Figure 4B, lanes 17–20). MTERF1 can thus efficiently block DNA unwinding by gp4.

We observed that the MTERF1-dependent block of DNA unwinding was not complete, since there was a weak, but noticeable increase in DNA unwinding at later time points, even in the presence of MTERF1 (Figure 4B, compare lanes 6 and 10). To investigate the possibility that MTERF1 stimulates helicase pausing rather than termination, we performed a more detailed time-course experiment using the DNA helicase substrate with the MTERF1 binding site in both forward (Figure 5A) and reverse direction (Figure 5B). In the forward direction, the addition of MTERF1 did not affect the DNA unwinding rate (Figure 5A and C). In the reverse direction, i.e. that corresponding to TWINKLE approaching MTERF1 in a direc-

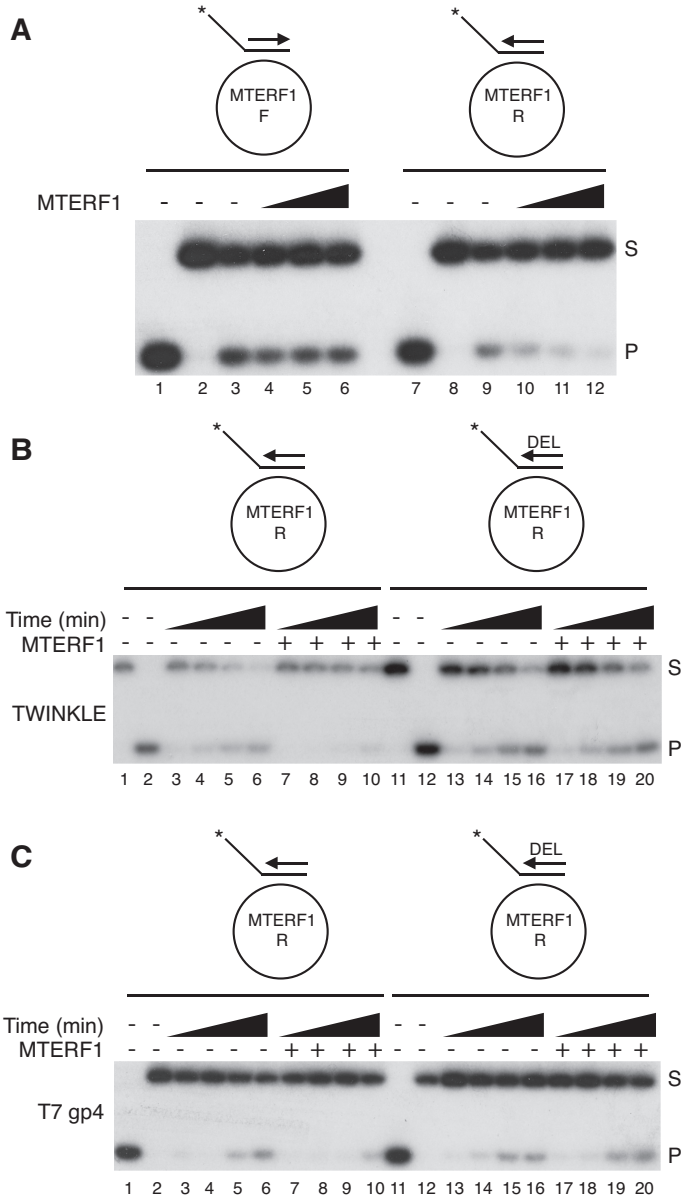

Figure 4. MTERF1 has contrahelicase activity. MTERF1 blocks TWINKLE dependent DNA unwinding in one direction, but not the other. (**A**) Helicase assays were performed as described in 'Materials and Methods'. The arrows show the directionality of the MTERF1 binding site; the star indicates the radioactivity-labeling site of the oligonucleotide. Lanes 1 and 7, substrate heated to 100°C before loading; lanes 2 and 8, untreated substrate. Increasing amounts of MTERF1 (100, 250 and 500 fmol) were added as indicated. (**B**) Time-course experiment using a helicase substrate with MTERF1 binding site in reverse direction or with a mutated MTERF1 binding site (4-bp deletion). Lanes 1 and 11, untreated substrate; lanes 2 and 12, substrate heated to 100°C before loading. TWINKLE (600 fmol) is added to lanes 3–10 and 13–20. (**C**) As in B, but with T7 gp4 instead of TWINKLE. Lanes 1 and 11, substrate heated to 100°C before loading; lane 2 and 12, untreated substrate. MTERF1 (500 fmol) was added to lanes 7–10 and lanes 17–20. T7 gp4 protein (100 fmol) was added to lanes 3–10 and 13–20. *S*, double-stranded substrate; *P*, single-stranded product.

tion opposite that of rDNA transcription, MTERF1 inhibited DNA unwinding. Interestingly, MTERF1 did not completely block TWINKLE-dependent unwinding, instead slowed it down significantly (Figure 5B and D). We repeated the experiment with the gp4 helicase and obtained similar results (data not shown). Based on these observations we conclude that MTERF1 is a potent contrahelicase that delays TWINKLE-dependent DNA unwinding in a direction-dependent way.

MTERF1 stimulates TWINKLE pausing *in vivo*

A previous study has demonstrated that overexpression of MTERF1 can increase replication pausing at MTERF1 binding sites *in vivo* (41). To investigate if the observed effect correlates with increased TWINKLE pausing *in vivo*, we performed chromatin immunoprecipitation (ChIP) using seven different primer pairs that covered the MTERF1 binding site and surrounding regions. In cells over-expressing a C-terminally Myc epitope-tagged

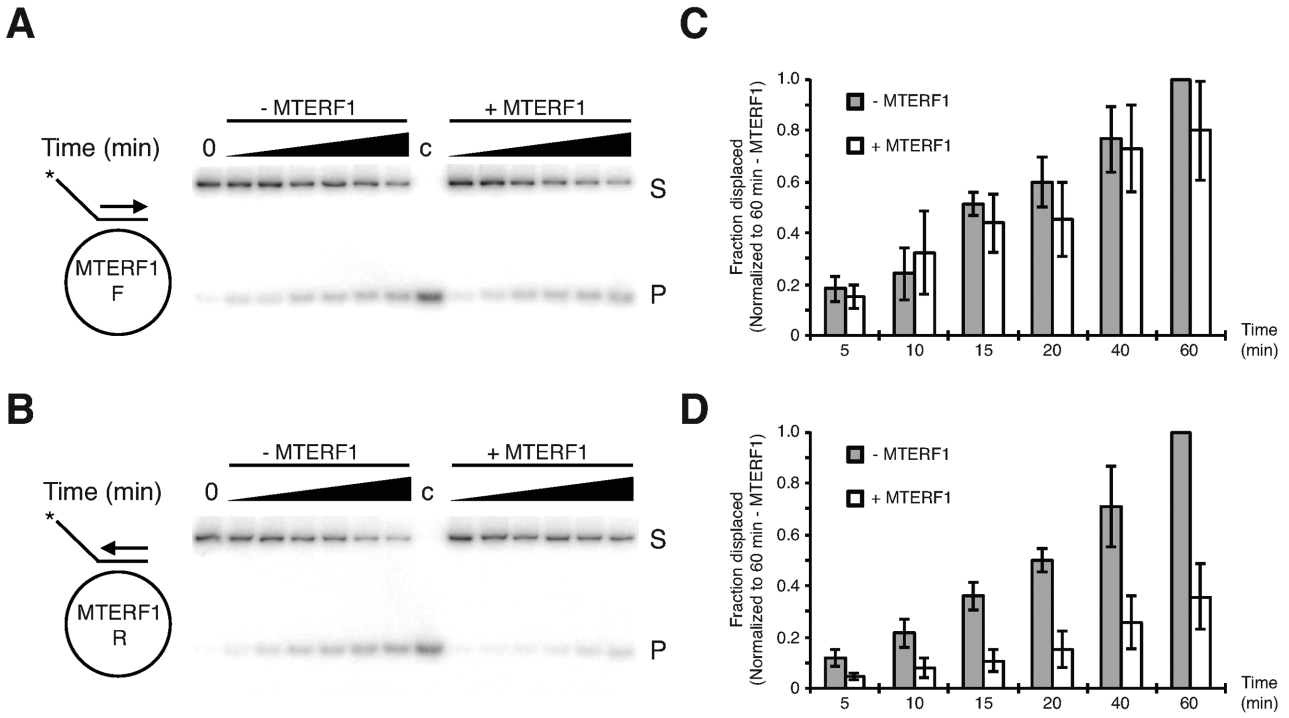

Figure 5. MTERF1 stimulates DNA helicase pausing, not termination. (**A** and **B**) DNA unwinding was measured in a time-course experiment in the absence or presence of MTERF1 (500 fmol), using two different substrates with the MTERF1 binding site in forward (A) or reverse orientation (B). (**C** and **D**) DNA unwinding efficiency was quantified from phosphorimager images for the substrate with the MTERF1 binding in the forward (C) or reverse (D) orientation. All results were normalized to the results of 60 min unwinding in the absence of MTERF1 for the respective template.

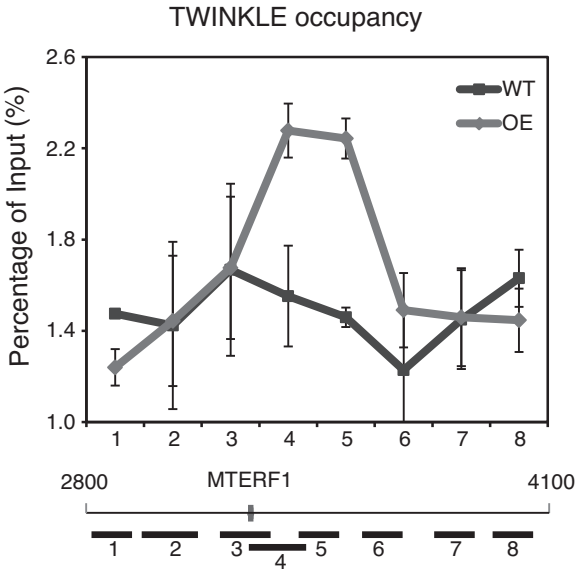

Figure 6. ChIP analysis of TWINKLE occupancy. ChIP analysis of TWINKLE occupancy in non-induced cells (dark grey) or cells overexpressing MTERF1 (light grey). The locations of PCR fragments used to monitor TWINKLE occupancy are indicated, the red box corresponds to the MTERF1 binding site.

MTERF1, we noted a peak of TWINKLE protein adjacent to the MTERF1 binding site (Figure 6). The peak was barely noticeable in the non-induced control cells. The results confirm that MTERF1 can increase TWINKLE occupancy levels at the MTERF1 binding site, hence supporting the idea that MTERF1 acts as a contrahelicase, inducing TWINKLE pausing also *in vivo*.

DISCUSSION

DNA replication and transcription are fundamental processes that must be performed with high fidelity. The enzymes responsible, DNA polymerase and RNA polymerase, act in coordination with a number of additional factors and travel for long distances on the DNA template. To the best of our knowledge, DNA replication and transcription in mitochondria are not separated in time, which means that these enzymatic machineries may use the same DNA template and therefore have the potential to interfere with each other. Studies in many different systems have demonstrated that head-on collision between transcription and replication can cause decreased replication fork stability, leading to replication stress (38). This helps to explain why highly transcribed genes are associated with increased levels of DNA mutations (transcription associated mutations, TAM) and recombination (transcription associated recombination, TAR) (44–47). Head-on collision may also affect the many proteins associated with co-transcriptional RNA processing, which in mitochondria include the precise endonucleolytic cleavage of the polycistronic transcripts. Bacterial genomes seem to have evolved to minimize the problem of replication - transcription collision. For instance, in *Escherichia coli*, the seven highly transcribed ribosomal

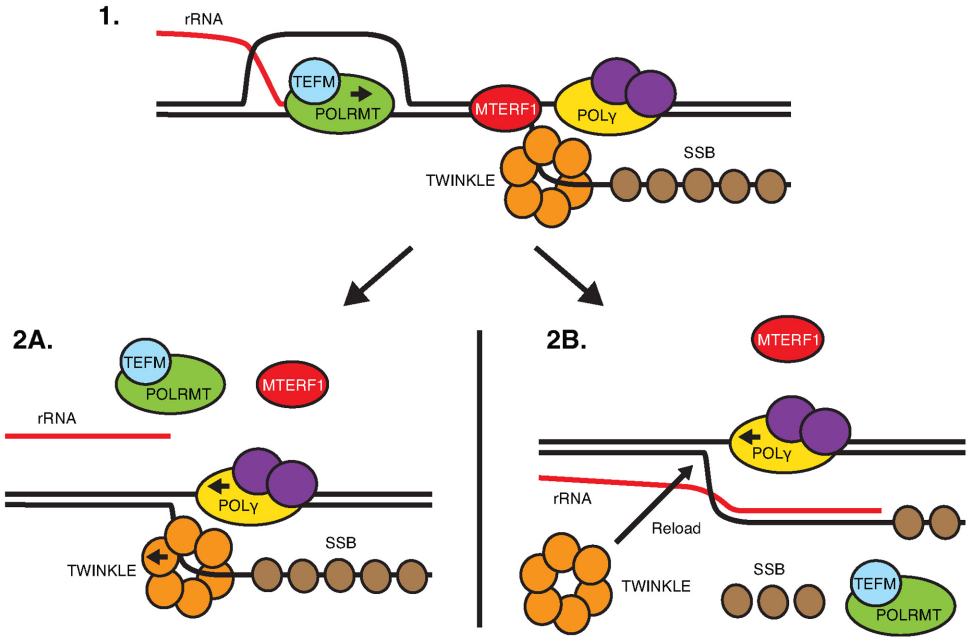

Figure 7. A schematic figure for the potential outcome of a collision between transcription and DNA replication and the MTERF1 binding site. **Step 1.** The mitochondrial replisome pauses at the MTERF1 binding site. Transcription initiated from HSP approaches the MTERF1 binding site during transcription of the rDNA region. **Alternative Step 2A.** Transcription displaces MTERF1 from the template, but is terminated immediately after when POLRMT collides with TWINKLE encircling the template strand. **Alternative Step 2B.** Transcription displaces MTERF1 and TWINKLE from the template strand. POLRMT continues to transcribe for another 25–75 nt before it terminates due to lack of processivity on ssDNA. TWINKLE is reloaded before H-strand DNA synthesis can be resumed.

genes are mainly present in regions near the origin of replication (48), oriented in a way so that replication and transcription take place in the same direction (49). Another means by which collisions between replication and transcription are prevented is via specific fork-progression barriers found at highly transcribed rDNA in eukaryotic nuclei (40).

Similar to fork-barrier proteins in many other systems, MTERF1 binds immediately downstream of the rDNA transcription unit (21). Previous reports have demonstrated that MTERF1 blocks antisense transcription across the rDNA region in both human and mouse mtDNA (24,25,27). As demonstrated here, MTERF1 can also arrest the moving replication with the same directionality as that observed for transcription. MTERF1 thus acts as a replication fork barrier and causes a delay in the progression of DNA replication in the direction opposite that of rDNA transcription. As a consequence, MTERF1 can act to avoid or manage head-on collisions between replication and transcription machineries in the rDNA region, thereby ensuring that POLRMT has completed synthesis of highly structured rRNA molecules before collision with the mtDNA synthesis machinery. Our findings also provide a biochemical explanation for the observation that overexpression of MTERF1 causes replication pausing *in vivo* (41). The effect is explained by the ability of MTERF1 to function as a contrahelicase with distinct polarity. In support of this notion, we employed 'chromatin' immunoprecipitation to demonstrate that MTERF1 stimulates TWINKLE pausing *in vivo.*

It is tempting to speculate that MTERF1 supports a process whereby POLRMT completes rRNA transcription initiated from HSP before H-strand DNA replication continues over the rDNA region. According to this model, the mitochondrial replisome pauses at the MTERF1 binding site (Figure 7, step 1). Once POLRMT has passed the rDNA region, transcription would facilitate the displacement MTERF1 and the block of mtDNA replication in the opposite direction is removed (Figure 7, step 2). This model predicts that POLRMT encounters the paused TWINKLE helicase, which encircles the transcribed strand. The most plausible outcome of this collision is that POLRMT terminates transcription and that the H-strand DNA synthesis is resumed (Figure 7, step 2A). This finding would be in line with findings in many other systems, where transcription normally yields to a moving replisome (38). A similar concept has been proposed for sea urchin (50,51) and Drosophila (52) mtDNA. In fact, the sea urchin mtDBP protein functions as a contrahelicase, inasmuch as it blocks the helicase activity of the heterologous simian virus 40 large T antigen (50).

Potentially, the interaction with MTERF1 weakens that of POLRMT and the template, which allows TWINKLE to displace POLRMT from the template strand. Termination of transcription in this manner would be a rare event, unlikely to significantly affect the relative levels of rRNA and mRNA molecules produced downstream of the MTERF1 binding site. The alternative outcome (Figure 7, step 2B) appears less likely. If POLRMT displaces TWINKLE it would entail replisome reloading after passage of the transcription machinery, a process that could lead to mispriming and increased levels of mutations. In addition, POLRMT has very low processivity on ssDNA and, if it managed to displace

TWINKLE, the polymerase would only continue transcribing for another 25–75 nt before termination (42).

What could be the consequences of unregulated, head-on collisions in the mitochondrial rDNA region? One possibility is that active replication can impair rRNA processing and maturation. Arguing against this possibility, characterization of the *Mterf1* knockout mice has not demonstrated any major effects on rRNA biogenesis (25). We instead favour the idea that MTERF1 blockage of DNA replication prevents transcription associated mutations or genetic alterations in mtDNA. Given the presence of thousands of mtDNA molecules in each mammalian cell, functional consequences of increased mutation levels and/or genetic stability may be difficult to detect in a short time span, which could explain the complete lack of observable phenotypes in the *Mterf1* knockout mice model. Nevertheless, the maintenance of mitochondrial genomic fidelity over evolutionary time must be sufficiently important for species survival, at least within the mammalian lineage where MTERF1, as well as its binding site, are conserved (53). In future experiments, we will develop tools to specifically identify effects on low levels genetic alterations in these mice, to see if we can detect increased levels of e.g. mtDNA mutations, which may corroborate this hypothesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank the Mammalian Protein Expression (MPE) Core Facility at the University of Gothenburg for cell production.

FUNDING

Swedish Research Council grants [2013-3621 to M.F., 2012-2583 to C.M.G.]; Swedish Cancer Foundation (to M.F. and C.M.G.); European Research Council [261248 to M.F., 268897 to C.G.]; IngaBritt and Arne Lundberg foundation (to M.F.); Knut and Alice Wallenbergs foundation (to M.F. and C.M.G.); Academy of Finland (FinMIT Centre of Excellence 118654 and Professorship 256615 to H.T.J.). Funding for open access charge: European Research Council.
Conflict of interest statement. None declared.

REFERENCES

1. Clayton,D.A. (1982) Replication of animal mitochondrial DNA. *Cell*, **28**, 693–705.
2. Falkenberg,M., Larsson,N.G. and Gustafsson,C.M. (2007) DNA replication and transcription in mammalian mitochondria. *Annu. Rev. Biochem.*, **76**, 679–699.
3. Yakubovskaya,E., Chen,Z., Carrodeguas,J.A., Kisker,C. and Bogenhagen,D.F. (2006) Functional human mitochondrial DNA polymerase gamma forms a heterotrimer. *J. Biol. Chem.*, **281**, 374–382.
4. Kaguni,L.S. (2004) DNA polymerase gamma, the mitochondrial replicase. *Annu. Rev. Biochem.*, **73**, 293–320.
5. Spelbrink,J.N., Li,F.Y., Tiranti,V., Nikali,K., Yuan,Q.P., Tariq,M., Wanrooij,S., Garrido,N., Comi,G., Morandi,L. *et al.* (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.*, **28**, 223–231.
6. Korhonen,J.A., Gaspari,M. and Falkenberg,M. (2003) TWINKLE Has 5' ->3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J. Biol. Chem.*, **278**, 48627–48632.
7. Shutt,T.E. and Gray,M.W. (2006) Twinkle, the mitochondrial replicative DNA helicase, is widespread in the eukaryotic radiation and may also be the mitochondrial DNA primase in most eukaryotes. *J. Mol. Evol.*, **62**, 588–599.
8. Milenkovic,D., Matic,S., Kuhl,I., Ruzzenente,B., Freyer,C., Jemt,E., Park,C.B., Falkenberg,M. and Larsson,N.G. (2013) TWINKLE is an essential mitochondrial helicase required for synthesis of nascent D-loop strands and complete mtDNA replication. *Hum. Mol. Genet.*, **22**, 1983–1993.
9. Tiranti,V., Rocchi,M., DiDonato,S. and Zeviani,M. (1993) Cloning of human and rat cDNAs encoding the mitochondrial single-stranded DNA-binding protein (SSB). *Gene*, **126**, 219–225.
10. Genuario,R. and Wong,T.W. (1993) Stimulation of DNA polymerase gamma by a mitochondrial single-strand DNA binding protein. *Cell. Mol. Biol. Res.*, **39**, 625–634.
11. Farr,C.L., Wang,Y. and Kaguni,L.S. (1999) Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein. Template-primer DNA binding and initiation and elongation of DNA strand synthesis. *J. Biol. Chem.*, **274**, 14779–14785.
12. Korhonen,J.A., Pham,X.H., Pellegrini,M. and Falkenberg,M. (2004) Reconstitution of a minimal mtDNA replisome in vitro. *EMBO J.*, **23**, 2423–2429.
13. Falkenberg,M., Gaspari,M., Rantanen,A., Trifunovic,A., Larsson,N.G. and Gustafsson,C.M. (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat. Genet.*, **31**, 289–294.
14. Metodiev,M.D., Lesko,N., Park,C.B., Camara,Y., Shi,Y., Wibom,R., Hultenby,K., Gustafsson,C.M. and Larsson,N.G. (2009) Methylation of 12S rRNA is necessary for in vivo stability of the small subunit of the mammalian mitochondrial ribosome. *Cell Metab.*, **9**, 386–397.
15. Sologub,M., Litonin,D., Anikin,M., Mustaev,A. and Temiakov,D. (2009) TFB2 is a transient component of the catalytic site of the human mitochondrial RNA polymerase. *Cell*, **139**, 934–944.
16. Shi,Y., Dierckx,A., Wanrooij,P.H., Wanrooij,S., Larsson,N.G., Wilhelmsson,L.M., Falkenberg,M. and Gustafsson,C.M. (2012) Mammalian transcription factor A is a core component of the mitochondrial transcription machinery. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 16510–16515.
17. Minczuk,M., He,J., Duch,A.M., Ettema,T.J., Chlebowski,A., Dzionek,K., Nijtmans,L.G., Huynen,M.A. and Holt,I.J. (2011) TEFM (c17orf42) is necessary for transcription of human mtDNA. *Nucleic Acids Res.*, **39**, 4284–4299.
18. Agaronyan,K., Morozov,Y.I., Anikin,M. and Temiakov,D. (2015) Mitochondrial biology. Replication-transcription switch in human mitochondria. *Science*, **347**, 548–551.
19. Posse,V., Shahzad,S., Falkenberg,M., Hallberg,B.M. and Gustafsson,C.M. (2015) TEFM is a potent stimulator of mitochondrial transcription elongation in vitro. *Nucleic Acids Res.*, **43**, 2615–2624.
20. Fuste,J.M., Wanrooij,S., Jemt,E., Granycome,C.E., Cluett,T.J., Shi,Y., Atanassova,N., Holt,I.J., Gustafsson,C.M. and Falkenberg,M. (2010) Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication. *Mol. Cell*, **37**, 67–78.
21. Fernandez-Silva,P., Martinez-Azorin,F., Micol,V. and Attardi,G. (1997) The human mitochondrial transcription termination factor (mTERF) is a multizipper protein but binds to DNA as a monomer, with evidence pointing to intramolecular leucine zipper interactions. *EMBO J.*, **16**, 1066–1079.
22. Christianson,T.W. and Clayton,D.A. (1988) A tridecamer DNA sequence supports human mitochondrial RNA 3'-end formation in vitro. *Mol. Cell. Biol.*, **8**, 4502–4509.
23. Martin,M., Cho,J., Cesare,A.J., Griffith,J.D. and Attardi,G. (2005) Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. *Cell*, **123**, 1227–1240.
24. Hyvarinen,A.K., Kumanto,M.K., Marjavaara,S.K. and Jacobs,H.T. (2010) Effects on mitochondrial transcription of manipulating mTERF protein levels in cultured human HEK293 cells. *BMC Mol. Biol.*, **11**, 72.

25. Terzioglu,M., Ruzzenente,B., Harmel,J., Mourier,A., Jemt,E., Lopez,M.D., Kukat,C., Stewart,J.B., Wibom,R., Meharg,C. *et al.* (2013) MTERF1 binds mtDNA to prevent transcriptional interference at the light-strand promoter but is dispensable for rRNA gene transcription regulation. *Cell Metab.*, **17**, 618–626.
26. Shang,J. and Clayton,D.A. (1994) Human mitochondrial transcription termination exhibits RNA polymerase independence and biased bipolarity in vitro. *J. Biol. Chem.*, **269**, 29112–29120.
27. Asin-Cayuela,J., Schwend,T., Farge,G. and Gustafsson,C.M. (2005) The human mitochondrial transcription termination factor (mTERF) is fully active in vitro in the non-phosphorylated form. *J. Biol. Chem.*, **280**, 25499–25505.
28. Robberson,D.L., Kasamatsu,H. and Vinograd,J. (1972) Replication of mitochondrial DNA. Circular replicative intermediates in mouse L cells. *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 737–741.
29. Miralles Fuste,J., Shi,Y., Wanrooij,S., Zhu,X., Jemt,E., Persson,O., Sabouri,N., Gustafsson,C.M. and Falkenberg,M. (2014) In vivo occupancy of mitochondrial single-stranded DNA binding protein supports the strand displacement mode of DNA replication. *PLoS Genet.*, **10**, e1004832.
30. Yasukawa,T., Reyes,A., Cluett,T.J., Yang,M.Y., Bowmaker,M., Jacobs,H.T. and Holt,I.J. (2006) Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *EMBO J.*, **25**, 5358–5371.
31. Pohjoismaki,J.L., Holmes,J.B., Wood,S.R., Yang,M.Y., Yasukawa,T., Reyes,A., Bailey,L.J., Cluett,T.J., Goffart,S., Willcox,S. *et al.* (2010) Mammalian mitochondrial DNA replication intermediates are essentially duplex but contain extensive tracts of RNA/DNA hybrid. *J. Mol. Biol.*, **397**, 1144–1155.
32. Reyes,A., Kazak,L., Wood,S.R., Yasukawa,T., Jacobs,H.T. and Holt,I.J. (2013) Mitochondrial DNA replication proceeds via a 'bootlace' mechanism involving the incorporation of processed transcripts. *Nucleic Acids Res.*, **41**, 5837–5850.
33. Tapper,D.P. and Clayton,D.A. (1981) Mechanism of replication of human mitochondrial DNA. Localization of the 5' ends of nascent daughter strands. *J. Biol. Chem.*, **256**, 5109–5115.
34. Brown,T.A., Cecconi,C., Tkachuk,A.N., Bustamante,C. and Clayton,D.A. (2005) Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. *Genes Dev.*, **19**, 2466–2476.
35. Kang,D., Miyako,K., Kai,Y., Irie,T. and Takeshige,K. (1997) In vivo determination of replication origins of human mitochondrial DNA by ligation-mediated polymerase chain reaction. *J. Biol. Chem.*, **272**, 15275–15279.
36. Wanrooij,S., Miralles Fuste,J., Stewart,J.B., Wanrooij,P.H., Samuelsson,T., Larsson,N.G., Gustafsson,C.M. and Falkenberg,M. (2012) In vivo mutagenesis reveals that OriL is essential for mitochondrial DNA replication. *EMBO Rep.*, **13**, 1130–1137.
37. Macao,B., Uhler,J.P., Siibak,T., Zhu,X., Shi,Y., Sheng,W., Olsson,M., Stewart,J.B., Gustafsson,C.M. and Falkenberg,M. (2015) The exonuclease activity of DNA polymerase gamma is required for ligation during mitochondrial DNA replication. *Nat. Commun.*, **6**, 7303.
38. Brambati,A., Colosio,A., Zardoni,L., Galanti,L. and Liberi,G. (2015) Replication and transcription on a collision course: eukaryotic regulation mechanisms and implications for DNA stability. *Front. Genet.*, **6**, 166.
39. Gerber,J.K., Gogel,E., Berger,C., Wallisch,M., Muller,F., Grummt,I. and Grummt,F. (1997) Termination of mammalian rDNA replication: polar arrest of replication fork movement by transcription termination factor TTF-I. *Cell*, **90**, 559–567.
40. Kobayashi,T. and Horiuchi,T. (1996) A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. *Genes Cells*, **1**, 465–474.
41. Hyvarinen,A.K., Pohjoismaki,J.L., Reyes,A., Wanrooij,S., Yasukawa,T., Karhunen,P.J., Spelbrink,J.N., Holt,I.J. and Jacobs,H.T. (2007) The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA. *Nucleic Acids Res.*, **35**, 6458–6474.
42. Wanrooij,S., Fuste,J.M., Farge,G., Shi,Y., Gustafsson,C.M. and Falkenberg,M. (2008) Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 11122–11127.
43. Pohjoismaki,J.L., Wanrooij,S., Hyvarinen,A.K., Goffart,S., Holt,I.J., Spelbrink,J.N. and Jacobs,H.T. (2006) Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells. *Nucleic Acids Res.*, **34**, 5815–5828.
44. Prado,F. and Aguilera,A. (2005) Impairment of replication fork progression mediates RNA polII transcription-associated recombination. *EMBO J.*, **24**, 1267–1276.
45. Kim,N., Abdulovic,A.L., Gealy,R., Lippert,M.J. and Jinks-Robertson,S. (2007) Transcription-associated mutagenesis in yeast is directly proportional to the level of gene expression and influenced by the direction of DNA replication. *DNA Repair (Amst.)*, **6**, 1285–1296.
46. Gottipati,P., Cassel,T.N., Savolainen,L. and Helleday,T. (2008) Transcription-associated recombination is dependent on replication in Mammalian cells. *Mol. Cell. Biol.*, **28**, 154–164.
47. Paul,S., Million-Weaver,S., Chattopadhyay,S., Sokurenko,E. and Merrikh,H. (2013) Accelerated gene evolution through replication-transcription conflicts. *Nature*, **495**, 512–515.
48. Ellwood,M. and Nomura,M. (1982) Chromosomal locations of the genes for rRNA in Escherichia coli K-12. *J. Bacteriol.*, **149**, 458–468.
49. Nomura,M. and Morgan,E.A. (1977) Genetics of bacterial ribosomes. *Annu. Rev. Genet.*, **11**, 297–347.
50. Polosa,P.L., Deceglie,S., Roberti,M., Gadaleta,M.N. and Cantatore,P. (2005) Contrahelicase activity of the mitochondrial transcription termination factor mtDBP. *Nucleic Acids Res.*, **33**, 3812–3820.
51. Roberti,M., Polosa,P.L., Bruni,F., Manzari,C., Deceglie,S., Gadaleta,M.N. and Cantatore,P. (2009) The MTERF family proteins: mitochondrial transcription regulators and beyond. *Biochim. Biophys. Acta*, **1787**, 303–311.
52. Joers,P., Lewis,S.C., Fukuoh,A., Parhiala,M., Ellila,S., Holt,I.J. and Jacobs,H.T. (2013) Mitochondrial transcription terminator family members mTTF and mTerf5 have opposing roles in coordination of mtDNA synthesis. *PLoS Genet.*, **9**, e1003800.
53. Linder,T., Park,C.B., Asin-Cayuela,J., Pellegrini,M., Larsson,N.G., Falkenberg,M., Samuelsson,T. and Gustafsson,C.M. (2005) A family of putative transcription termination factors shared amongst metazoans and plants. *Curr Genet.*, **48**, 265–269.